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SPINAL CORD GENES ENRICHED IN RAT DORSAL HORN AND INDUCED BY NOXIOUS STIMULATION IDENTIFIED BY SUBTRACTION CLONING AND DIFFERENTIAL HYBRIDIZATION

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Abstract—Persistent nociceptive input increases neuronal excitability and induces a program of gene expression in the dorsal spinal cord. The alteration in gene expression commences with phosphorylation and induction of immediate early genes and proceeds to target genes. Only a few target genes have been identified as yet. The present report uses a polymerase chain reaction-based subtraction cloning procedure to obtain an "anatomically focused" complementary DNA library enriched in transcripts related to sensory spinal cord (rat dorsal horn minus ventral horn). A subset of clones from this library ($n = 158$) was screened to verify dorsal horn enrichment and to identify those regulated by carrageenan-induced peripheral inflammation. Molecular classes which displayed enriched expression included a proto-oncogene not previously associated with sensory processes, two regulators of the Rho/Rac pathway which controls cell shape, and three genes involved in cytoskeletal regulation and scaffolding. Additional transcripts coded for proteins involved in intercellular communication or intracellular function. Within the set of 158 transcripts, one known and two unknown genes were induced by persistent noxious input. The known gene codes for the secreted cysteine proteinase inhibitor, cystatin C, suggesting that modulation of extracellular proteolytic activity occurs. Since it is secreted, cystatin C may also provide a cerebrospinal fluid bio-marker for persistent pain states.

Using a combined anatomical and functional approach, we have extended the molecular repertoire of genes expressed and induced in second-order neurons or supporting glial cells in several new directions, with particular emphasis on regulation of cell morphology and plasma membrane dynamics. Some of these proteins reveal new pathways for information signaling in the sensory half of the spinal cord and require further research to understand their role in the adult spinal cord. The induced genes may provide new molecular targets for therapeutic development and provide new probes for investigating the dynamic state of cellular activity that occurs during persistent pain states. Published by Elsevier Science Ltd on behalf of IBRO.

Key words: somatosensory, nociception, inflammation, pain, neuronal plasticity, cDNA subtraction.

The primary afferent innervation of second-order spinal cord neurons represents the first critical synaptic connection in somatosensory information transmission. For nociceptive processing, an acute painful stimulus is the simplest activation of this circuit and, depending on stimulus intensity, a reflex withdrawal can be provoked. Spinal cord neurons also relay information to the brain, where a network of regions related to sensory perception, motor integration and attention is activated.[4,13,21] Nociceptive functions in the acute situation become considerably more complex during persistent painful input. The second-order neurons exhibit a marked plasticity of neuronal physiology that involves neuronal hyperactivity and up-regulation of gene expression, some of which code for immediate early transcription proteins[12,35,52] and neuropeptides.[2,7,19,20,50] The opioid genes have been widely investigated following application of a variety of persistent nociceptive stimuli.[2,7,30,54] However, the range of molecular alterations remains largely unknown, which in turn hampers the ability to understand the mechanisms of transcriptional up-regulation in the nucleus and related upstream signal transduction pathways.

In the present report, we use subtraction cloning and differential hybridization to investigate which genes play a role in dorsal horn sensory processes. We hypothesized that a set of genes exhibits enriched expression in the dorsal horn and that subsets of these genes play important roles in dorsal horn somatosensory processes, nociception-induced plasticity and tissue-specific neurochemistry. A two-step system was developed to identify sets of these genes. First, an anatomically focussed subtractive cDNA library of dorsal spinal cord-specific genes was prepared. Over the past few years, several powerful approaches to subtraction cloning have become available.[16,18,27,38,51] Several of these utilize polymerase chain reaction (PCR) to amplify the differences between two genomes or sets of transcripts and suppress or block amplification of common sequences within the two sets. A "suppression-subtractive"

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Abbreviations: CGRP, calcitonin gene-related peptide; EDTA, ethylenediaminetetra-acetate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; RhoGAP, Rho GTPase-activating protein; RT, reverse transcriptase; SAP-97, synapse-associated protein 97; SSC, standard saline citrate.

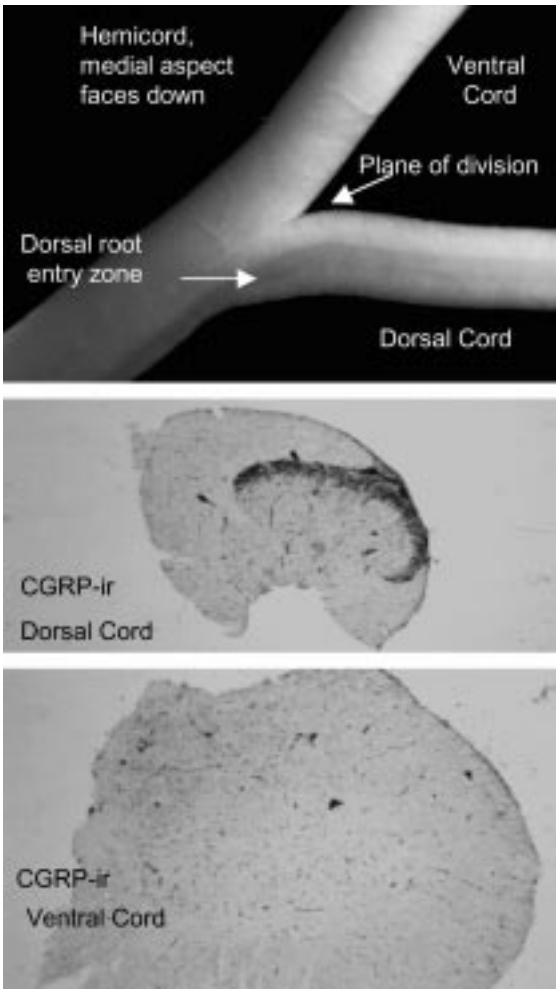

Fig. 1. Dissection of whole spinal cord into dorsal and ventral regions. The whole spinal cord was removed from the rat and dissected through laminae V/VI into dorsal and ventral parts (top panel). Middle and bottom panels: immersion fixed sections obtained from dorsal and ventral samples stained for CGRP. The dorsal part (middle panel) contained all of laminae I as shown by CGRP immunostaining, and also laminae II through IV. The ventral part (bottom panel) contains all of laminae VII–IX. The typical light CGRP immunostaining of motor neurons was not preserved well with the immersion fixation.

hybridization protocol[9,10] was selected for the present study because of its capacity to isolate low-abundance transcripts and to perform the subtraction in an iterative fashion. We subtracted ventral from dorsal spinal cord to remove housekeeping genes and other genes with a general neural or glial function. The subtraction product was cloned and representative cDNAs sequenced. We screened 158 of the sequences to verify their enrichment in the dorsal horn. In the second step, this pool was screened again to identify genes whose expression is induced by persistent noxious input during peripheral inflammation. The subtractive approach identifies several new genes induced by nociceptive synaptic activity and suggests several directions for dorsal horn signal transduction mechanisms related to structural plasticity that may accompany altered neuronal function during persistent pain states.

EXPERIMENTAL PROCEDURES

Dissection and isolation of total RNA and poly(A)$^+$ RNA

Male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were killed by decapitation, and whole spinal cords were immediately removed according to the method of DeSousa and Horrocks.[8] The dorsal horn and ventral horn samples were rapidly obtained as follows. The entire spinal cord was placed on a cold plate and dissected longitudinally into left and right halves by placing a midline cut at the sacral–lower lumbar level and pulling the two halves apart. Each half was placed medial side down. The entire rostrocaudal extent of the dorsal portion could be reproducibly removed by placing an initial blunt cut into the lateral convexity, again at the lower lumber level. The neck of the dorsal horn provides a natural plane of division and the cord divides readily at laminae V/VI into two parts, the superficial laminae and the ventral cord (Fig. 1A). The dorsal sample contained all of lamina I as shown by immunostaining freshly dissected, immersion-fixed samples for calcitonin gene-related peptide (CGRP) immunoreactivity (Fig. 1B). The sample also contains laminae II through IV, as seen in paraffin-embedded sections prepared with standard histological stains (e.g. hematoxylin–eosin, thionin; data not shown).

Total RNA was isolated from dorsal and ventral spinal cord using TRIzol reagent (Life Technologies, MD) according to the manufacturer's instructions. Poly(A)$^+$ RNAs were isolated from total RNA using the Fast Track 2.0 mRNA isolation kit (Invitrogen, San Diego, CA). This poly(A)$^+$ RNA was used for generation of the subtracted library. RNA content was quantified fluorometrically using the RiboGreen reagent (Molecular Probes, Eugene, OR) in a 96-well plate format; this method allows substantially greater conservation of the RNA extracted from small tissue samples or after poly(A)$^+$ purification than standard spectrophotometric methods.

Preparation of a subtracted library and subtraction efficiency

Suppression-subtractive hybridization[9] was performed between dorsal (tester) and ventral (driver) cord using the PCR-Select cDNA Subtraction kit according to the manufacturer's instructions (Clontech). The subtracted (dorsal cord minus ventral cord) cDNAs were assessed for subtraction efficiency by estimating the abundance of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in the cDNA pools before and after subtraction using PCR analysis. The PCR was performed for 18, 23, 28 and 33 cycles with a Stratagene Robocycler Gradient 96 Temperature cycler. PCR products were examined on a 2.0% agarose/ethidium bromide gel.

Cloning and sequence analysis

After the analysis of subtraction efficiency, the PCR-amplified, subtracted cDNAs (dorsal cord minus ventral cord) were inserted into pT-Adv using a TA cloning kit (Clontech, Advantage PCR Cloning kit). The subtracted cDNA library was plated onto 100 mm × 15 mm kanamycin (50 μg/ml) containing agar plates pretreated with isopropyl-β-D-thiogalactopyranoside (40 μl of 100 mM solution) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (1.6 mg). A total of 240 individual recombinant clones (totally white clones) were picked, grown in Terrific broth (Quality Biological, MD) containing kanamycin (50 μg/ml) and then used to prepare plasmids for DNA sequencing. DNA sequencing was carried out by the NIDCR Sequencing Core Facility using an ABI Dyedeoxy Terminator Cycle Sequencing apparatus (ABI Prism 377 DNA sequencer, Applied Biosystems). The sequences of the cDNA inserts were compared with the GenBank non-redundant and expressed sequence tag databases after stripping out vector and primer sequences.

In the course of generating the subtracted library, a non-subtracted library was also generated and we performed a limited amount of sequencing of cDNA clones from the non-subtracted library.

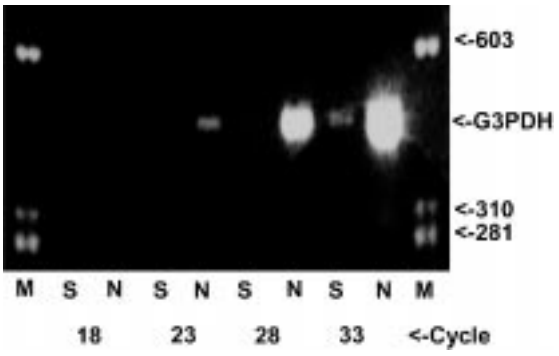

Fig. 2. Assessment of subtraction efficiency. PCR was performed on subtracted (lanes S) or non-subtracted (lanes N) cDNAs with the G3PDH primers. G3PDH PCR product was detected in the non-subtracted sample after 23 cycles, but not in the subtracted sample until 33 cycles. Lane M, molecular weight marker.

Hybridization screening of the subtracted cDNA library

A total of 158 clones (136 from the subtracted and 22 from the non-subtracted library) were selected based on the results of the sequence analysis to exclude the very short inserts and comparison with database sequences. Their inserts were amplified by PCR using the Clontech Advantage cDNA PCR kit. The amplified PCR products were examined on 2% agarose/ethidium bromide gels and the amount of DNA in each tube was quantified fluorometrically using the PicoGreen reagent (Molecular Probes, Eugene, OR). The PCR products which showed a single major band only were again selected, dissolved at 25 ng DNA/μl in denaturation solution and dotted onto nitrocellulose membranes with a four-place manual pin replicator; approximately 20 ng of DNA was spotted per dot. The membranes were washed in 2×standard saline citrate (SSC) for 5 min and then exposed to UV light to crosslink cDNAs to the membranes using the Stratalinker UV crosslinker (Stratagene, La Jolla, CA). Membranes were stored at −20°C until hybridization.

Hybridization screening was performed with [^{33}P]dCTP-labeled single stranded DNA probes prepared from total RNA and oligo $dT_{12\text{-}15\text{-}18}$ primer mixture using the Superscript II kit (Life Technologies, Gaithersburg, MD). For preparation of ^{33}P-labeled probes, the reaction mixture (in 50 μl of first strand buffer) contained 30 μg total RNA, which was pretreated at 70°C for 10 min and chilled on ice, 10 mM dithiothreitol, 150 μM each of dATP, dGTP and dTTP, 1.5 μM dCTP, 200 μCi [α-^{33}P]dCTP, 40 units RNasin, 500 ng $oligo(dT)_{12\text{-}15\text{-}18}$ primer, and 200 units reverse transcriptase (RT). The mixture was incubated at 42°C for 60 min and the reaction terminated by addition of 10 μl of 1 M NaOH, 5 mM EDTA and heating at 65°C for 1 h. To the reaction mixture, 25 μl of 10 M CH_3COONH_4 (pH 7) and 300 μl ethanol were added. The reaction mixture was centrifuged in a microfuge at maximum speed for 10 min after standing in dry ice for 1 h. The pellet was washed with 200 μl ethanol and then dissolved in 100 μl H_2O and used for hybridization.

For hybridization, membranes dotted with cDNA as described above were prehybridized in 2×SSC containing 1% sodium dodecylsulfate and sheared salmon sperm DNA (0.5 mg/ml) at 55°C for at least 4 h. The prehybridization buffer was discarded and the membranes were then hybridized with the same buffer mixture containing $2.5–10\times10^6$ c.p.m./ml of ^{33}P-labeled cDNA probe at 55°C overnight. Membranes were washed three times with 2×SSC containing 1% sodium dodecylsulfate at 55°C for 30 min and then placed on a phosphor screen or exposed to X-ray film with an intensifying screen at −80°C for one to four days.

Quantification of mRNA by ribonuclease protection assay

^{33}P-labeled RNA probes were generated with cDNA inserts ligated to SP6 and T3 promoters as templates and [α-^{33}P]CTP using the Riboprobe *in vitro* Transcription System (Promega, Madison, WI). Both strands of riboprobe were prepared from each cDNA. Ribonuclease protection assay was performed using the ribonuclease protection assay III kit from Ambion (Austin, TX) with slight modification. Briefly, after hybridization of the ^{33}P-labeled probe with RNA and RNase digestion, the hybridized RNA was precipitated and washed according to the instructions in the kit, and then counted in a scintillation counter.

Animals and carrageenan treatment

Male Sprague–Dawley rats (Harlan; 250–350 g) were housed with a 12-h light period and free access to food and water. Procedures for all animals used were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by the NICDR Animal Care and Use Committee. All efforts were made to minimize both animal numbers and suffering in the experiments.

Carrageenan Lambda type IV (Sigma, St. Louis, MO; 3 mg in 150 μl phosphate-buffered saline per paw) was injected bilaterally into the plantar surface of the hindpaw. Rats (16 per group) were killed 24 and 48 h after treatments and spinal cords were removed as described above. Segments of spinal cord (6–8 mm) containing L4 and L5 were dissected, separated into dorsal and ventral parts, frozen immediately in dry ice and stored at −80°C. The frozen tissue was processed for RNA extraction with TRIzol reagent as described above. The extracted RNAs were used as templates for preparation of ^{33}P-labeled cDNA probes used in the hybridization screening.

RESULTS

Subtraction efficiency

The subtraction efficiency was evaluated by comparing the abundance of G3PDH cDNA in the subtracted and non-subtracted cDNAs by PCR analysis. DNA concentrations in the subtracted and non-subtracted cDNAs were adjusted to be equal and then PCR was performed using G3PDH-specific primers. The analysis of the PCR products on an agarose/ethidium bromide gel is shown in Fig. 2. After 23 cycles, G3PDH cDNA was detected in the non-subtracted cDNA but not in the subtracted cDNA. G3PDH cDNA was detected in the subtracted cDNA after 33 cycles of PCR and the fluorescent intensity of this PCR product was about equal to that observed in non-subtracted cDNA after 23 cycles of PCR. The marked reduction in the abundance of G3PDH in the subtracted cDNA indicates that common housekeeping sequences were substantially removed by the suppressive subtraction procedure without over-subtracting for absolute differences.

Identification of dorsal spinal cord enriched cDNAs by hybridization screening and reverse transcriptase–polymerase chain reaction

To identify clones which are enriched in the dorsal spinal cord, cDNA inserts, including 129 from the subtracted library and 29 from the non-subtracted library, were individually amplified by PCR and dotted on nitrocellulose membranes. Two membranes were screened: one was hybridized with ^{33}P-labeled cDNA from dorsal spinal cord RNA and the other with ^{33}P-labeled cDNA from ventral spinal cord RNA. After hybridization, the membranes were placed on a phosphor storage screen and the results from the two membranes were compared visually (Fig. 3). The experiment was repeated with a

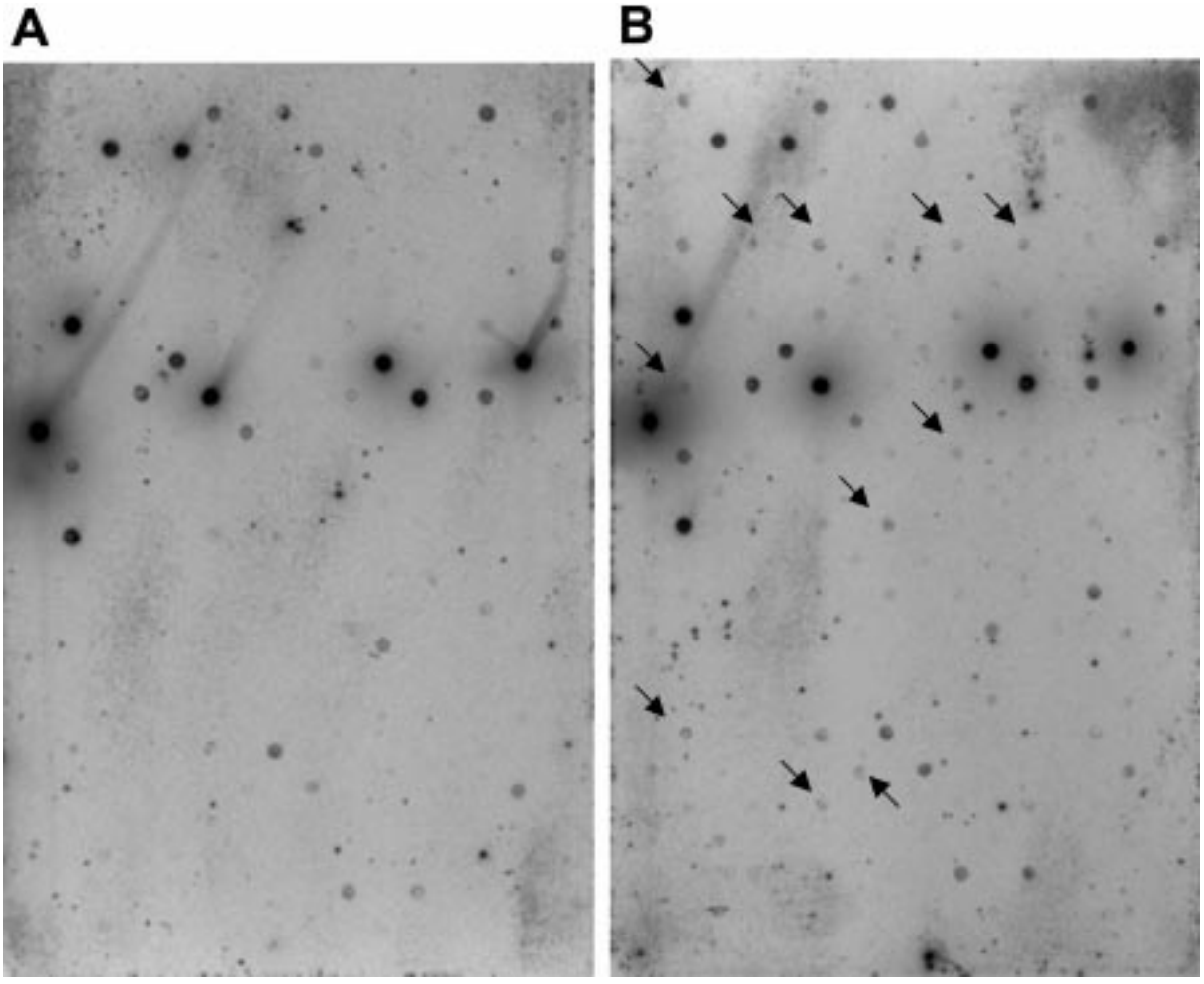

Fig. 3. Hybridization screening of clones from the subtracted cDNA library for dorsal cord enriched transcripts. A total of 158 cDNA inserts were amplified by PCR and approximately 20 ng of insert DNA was dotted onto nitrocellulose membranes. The membranes were then hybridized with ^{33}P-labeled cDNA probes from dorsal spinal cord RNA (B) and ventral spinal cord RNA (A). Typical examples of clones exhibiting an enriched level of dorsal spinal cord expression in the subtracted cDNA inserts are indicated by arrows.

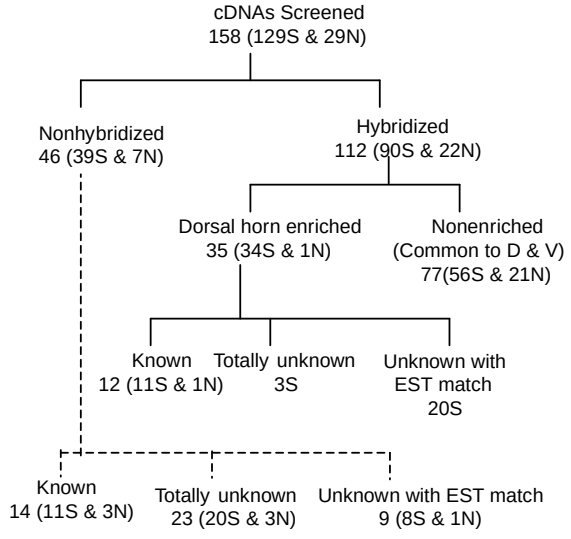

Fig. 4. Summary of the hybridization screening results in Fig. 3. The hybridization screening as described in Fig. 3 was repeated twice and the results are summarized. D, dorsal horn; V, ventral horn.

second pair of membranes and the results of these two independent experiments are summarized in Fig. 4. Under the hybridization conditions used, about 66% of the cDNA inserts (112 DNA dots including 90 subtracted and 22 non-subtracted clones) were hybridized and 46 cDNAs (39 subtracted and seven non-subtracted clones) were not hybridized. Thirty-five clones including three total unknowns, 20 uncharacterized transcripts with an expressed sequence tag match (to any of a wide variety of tissues and species) and 12 knowns were enriched in the dorsal cord. There were 29 cDNAs from the non-subtracted library included in the membrane and only one of these cDNAs was enriched in the dorsal spinal cord. The known clones found to be enriched in the dorsal spinal cord are listed in Table 1.

Further examination of dorsal spinal cord enriched cDNAs was performed by RT–PCR using RNA from both dorsal and ventral spinal cord as template. To confirm the results of membrane hybridization screening, two dorsal spinal cord enriched cDNAs, 41S (Fig. 5) and 52S, were subjected to RT–PCR analysis. After 24

Table 1. Dorsal spinal cord enriched known clones

1	23S	cDNA N14 encoding thymic stromal Ag HS9 a potent molecule participating in intrathymic T cell development (D67067)[47]
2	28S	Rat mRNA for $GABA_A$ receptor β3 subunit (X15468)[56]
3	41S	Shares DNA sequence homology to mouse 8.5 mRNA encoding a 19-kDa protein, expressed in neural and neuroendocrine tissues, localized in juxtanuclear Golgi-like structure (U17259; X82774)[41]
4	88S	Rat opioid-binding cell adhesion molecule (M79310)[26]
5	89S	*H. sapiens* mRNA for tre oncogene, detected in wide variety of human cancer cells but not in human cells from normal tissue (NM004505)[34]
6	170S	Lis-1 cDNA encoding the murine lissencephalyl-1 protein, the platelet-activating factor acetylhydrolase 1b α subunit (U95120)[37]
7	175S	Human mRNA encoding p97 subunit of the 26S proteasome, a polypeptide identical to the type 1 tumor necrosis factor receptor-associated protein-2/55.11. 26S. It might serve as a mediator molecule in the tumor necrosis factor signaling pathway in cells (X86446)[49]
8	244S	5′ end of cDNA is highly homologous to rat γ-adducin mRNA (U35775[3]), an *in vivo* substrate for protein kinase C[11] and Rho-activated kinase[14]
9	319S	Isoform of rat testis β-chimaerin mRNA (X69489), a RhoGAP[25] or a rat form of a human β-2-chimaerin-like clone (AK026415)
10	372S	Homologous to human mRNA for *dbl* proto-oncogene (AL033403), Rho GTP exchange factor[40]
11	373S	Mouse Erp99 (J03297), an abundant, conserved glycoprotein of endoplasmic reticulum, is homologous to the 90-kDa heat shock protein[29]
12	396S	Rat 97-kDa protein, SAP-97 (U14950)[33]

cycles of PCR, a small amount of PCR product from cDNA 41S was generated from the dorsal cord RNA, but not from the ventral cord RNA. After 27 cycles of PCR, the PCR products generated from dorsal spinal cord RNA were markedly increased in comparison to using ventral cord RNA as template. In contrast, equal amounts of β-actin PCR products were generated from both dorsal and ventral spinal cord RNA after 24 or 27 PCR cycles. For cDNA 52S, the predicted PCR product was detected after 27 cycles of PCR with the dorsal spinal cord RNA, but a barely detectable amount of PCR product was generated from ventral spinal cord RNA (Fig. 6). These RT–PCR results further confirm the results of the membrane hybridization screening that clones 41S and 52S are enriched in the dorsal spinal cord.

In the membrane hybridization screening analysis, 46 clones were not hybridized (Fig. 4), probably because of their low abundance in the tissue. In order to find a more sensitive method to study the low-abundance clones, nine of the 46 non-hybridized cDNA inserts were selected, specific PCR primers were prepared, and RT–PCR was performed using dorsal and ventral spinal cord RNA as templates. Two of these clones, 88S and 313S, were enriched in the dorsal cord (Fig. 6) and another seven clones were common to both dorsal and ventral cords.

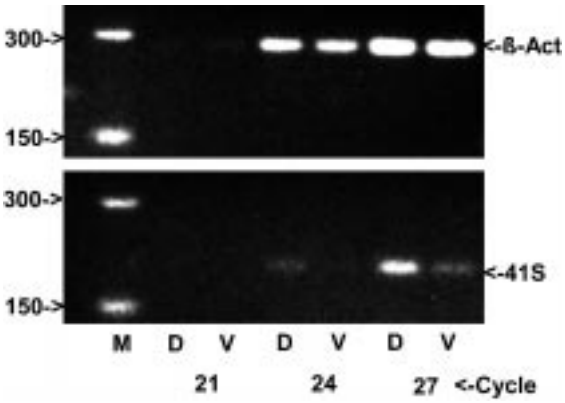

Fig. 5. Enrichment of 41S clone in the dorsal spinal cord as shown by RT–PCR. RT–PCR was performed with specific primers for 41S clone on total RNAs from dorsal (D) and ventral (V) spinal cords (bottom). The PCR was performed for 21, 24 and 27 cycles. For comparison, RT–PCR was also performed for β-actin (top). Lane M, molecular weight marker.

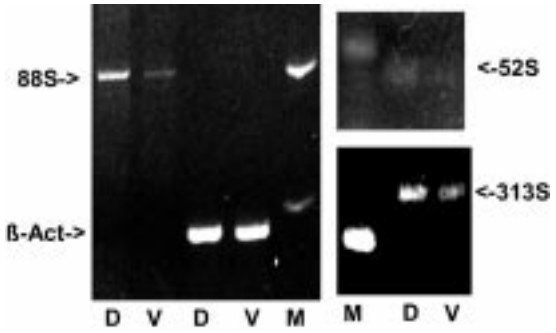

Fig. 6. Enrichment of 313S, 52S and 88S clones in the dorsal spinal cord as shown by RT–PCR. RT–PCR was performed with specific primers for 52S (right top), 313S (right bottom) and 88S (left) on total RNA from dorsal (D) and ventral (V) spinal cord samples. PCR was performed for 27 cycles. In each case, the transcript level is dorsally enriched. For comparison, RT–PCR was also performed for β-actin (bottom of left panel). Lane M, molecular weight marker.

Identification of clones which are regulated during carrageenan inflammation

To search for genes regulated by noxious stimuli, rats were treated with carrageenan, and ^{33}P-labeled cDNA probes were prepared from dorsal cord RNA extracted at 24 and 48 h of peripheral inflammation induced by carrageenan and from control non-inflamed rats. Fresh membranes containing the 158 cDNAs (described above) were hybridized with the ^{33}P-labeled cDNA probes from the carrageenan-treated or control animals. The hybridization screening was repeated twice and nine clones (306S, 324S, 29N, 191S, 41S, 93S, 39N, 354S and 362S) were found to be altered by the carrageenan-induced inflammation. Typical signals from the membrane are shown in Fig. 7A. In order to confirm the results of hybridization screening with a second,

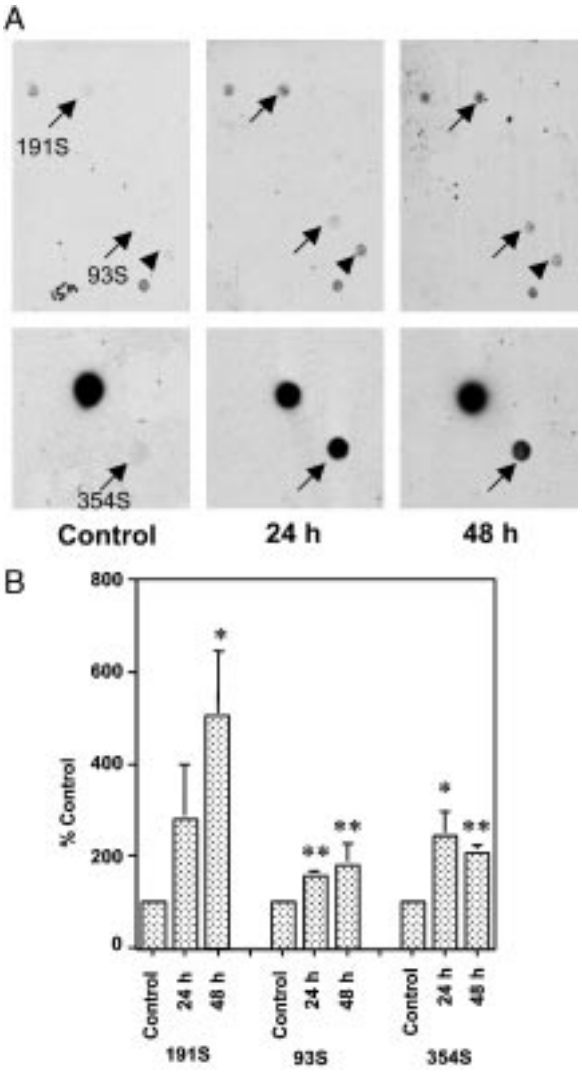

Fig. 7. (A) Hybridization screening for carrageenan inflammation up-regulated genes. Rats were treated with phosphate-buffered saline (control) and carrageenan (3 mg in 150 μl buffered saline) bilaterally into the plantar surface of the hindpaw and killed 24 and 48 h after treatments. Segments of spinal cord were dissected out from the L4 and L5 regions, and separated into dorsal and ventral parts. RNA was extracted from the dorsal cords and used to prepare ^{33}P-labelled single stranded DNA probes for hybridization screening. The membranes, dotted with 158 cDNA inserts as described in Fig. 3, were hybridized with the ^{33}P-labelled probes. Clones 191S, 93S and 354S (arrows) were increased in the carrageenan-treated rats. The arrowhead near 93S shows one clone that was not reliably elevated by carrageenan inflammation in the replicate hybridizations. (B) Up-regulation of 191S, 93S and 354S clones by carrageenan inflammation as shown by ribonuclease protection assay. The RNAs from control and carrageenan-treated rats as described in Fig. 7A were analyzed by ribonuclease protection assay and results were expressed by setting the control as 100%. The control c.p.m. values for 191S ($n=3$), 93S ($n=3$) and 354S ($n=3$) were 1028 ± 137.6, 716.7 ± 27.14 and 2990.0 ± 558.4, respectively. Statistical analyses were performed on the c.p.m. data and comparisons made by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.005$.

independent method, these nine clones were further analyzed by ribonuclease protection assay. The results are shown in Fig. 7B.

For the ribonuclease protection assay, RNA was prepared from the dorsal spinal cord of carrageenan-treated and control rats and then subjected to ribonuclease protection assay analysis. Clones 191S, 93S and 354S were found to be increased by carrageenan-induced noxious stimuli, confirming the results of membrane screening hybridization (Fig. 7A). In contrast, three clones, 29N, 41S and 39N, were found not to be altered by the carrageenan treatment (i.e. the results of the membrane hybridization on these three clones were not confirmed); for the remaining three clones the results were inconclusive. The cDNA sequences of 191S and 93S are shown in Fig. 8A.

DISCUSSION

In the present study, a library enriched with dorsal spinal cord cDNAs was generated using the suppression-subtractive hybridization technique to identify genes involved in sensory processes and altered gene expression during noxious stimulation. The subtraction efficiency, based on PCR of G3PDH, is estimated to be about 400-fold assuming that five PCR cycles corresponds to ~20-fold enrichment (Clontech PCR-Select cDNA Subtraction Kit Manual). When this library was further screened for dorsal cord-specific cDNAs using membrane hybridization with ^{33}P-labeled probes prepared from dorsal or ventral cord RNAs, 90 of 129 subtracted cDNAs were hybridized (Fig. 3). Among the hybridized cDNAs, 38% (34/90 × 100) were found to be enriched in the dorsal spinal cord. RT–PCR using specific primers further confirmed the dorsal enrichment of two randomly picked dorsal cord clones, 41S and 52S (Fig. 4). Twenty-nine cDNAs from the non-subtracted library were also screened, but here only one clone of 22 non-subtracted cDNAs gave a signal that was dorsally enriched (i.e. 5% of hybridized, non-subtracted). The higher representation of dorsal spinal cord-enriched transcripts in the subtracted cDNA library further confirms the success of this strategy.

Although only approximately 38% of the subtracted cDNAs detected were enriched in the dorsal horn, many of the genes were novel (Fig. 4). Thus, dorsal–ventral anatomical subtraction provides a powerful approach for identifying novel genes involved in somatosensory processing and persistent pain states. We intentionally chose to under-subtract rather than aim for a complete dichotomy since we (i) did not want to exclude genes with a minor degree of ventral horn expression but a preponderance of expression in the dorsal horn [for example, clone 28S in Table 1 ($GABA_A$ receptor β3 subunit)], and (ii) wanted to use the library for screening pain state-specific up-regulation. In some subtraction studies, the conditions for subtraction were stringent, resulting in only a limited set of genes.[32] In our study and those of others,[38] the subtracted library is screened post hoc with differential hybridization, allowing the potential for detection of genes exhibiting less extreme differential expression. For example, $GABA_A$ receptor β3 subunit (clone 28S in Table 1) is preferentially expressed in the dorsal horn and, as reported by Wisden *et al.*,[53] it is highest in the dorsal horn but is also moderately expressed in motor neurons. Interestingly, a

A **93S**

ACAAGCTCCAATACAGATACTATTTACCAATACAGGTAAGTAGCAAACCTAACCAGCAAGTATTTGAGTAT
CCACAAAAGGCATTACAAAAGCCGTGAGACTATGGTTCTATTTATTGCCTTGAGTAATGCACTAAAAGCAG
GAAGATGAAAAGATGATACAGGCGTTCCTGGGGGGAGACAGACTGCCTTGAAAGCACAGGATTATGCTTCT
AACAACCTTAGCTCAAAACACTAATACTGTGGCAGTTATCGGCTATCAAAT

191S

TACTGTTTGTATTACTTTGTTGTTTCTTTAAAAGTCACTTATTTGCTTTTACTGAAGTTAAATGCTGGGGC
AGGCATCATGAACACTTGTAATTCTGTGGTATCTANGTATTAAGTGTAGCAGAGAATAATAAAAAAGATTT
AGCTTCCTACACGGGTGGGCCATATGGCACCAGCAAGGTGTTCTCTCGGTAGACCTCTAACCCTTTGTTCT
CAAAATGTTTCCACCCGACTAGCTAAGTTCCCGTGCAGGTTGGT

B **244S**

CATGGCACAGAGACAGCAGCGTGAAAAAACAAGATGGCTGAATTCTCCAAATACATACATGAAAGTGAATG
ATGCCGGAGGAGTCCCGGAACGGGGAAACTAGTCCCAGGACCAAAATAACGTGGATGAAAGCAGAGGACCC
CTCTAAAGTTAGTAGTGGAACACCTATCAAAATTGAAGATCCCAATCAGTTTGTTCCTCTAAACACAAACC
CTTGTGTCGAAGGCTGACTTTCAATAGATCGCAGCGAGGGAGCTGCTCTGCTACGT

Comparison of γ-adducin with translated result of clone 244S

```
           428
γ-adducin   MAQ RQQREKTRWL NSPNTYMKVN VPEESRNGET SPRTKITWMK AEDPSKVSSG
Clone 244S  MAQ RQQREKTRWL NSPNTYMKVN VPEESRNGET SPRTKITWMK AEDPSKVSSG
                                  502
γ-adducin   TPIKIEDPNQ FVPLNTNPTE VLEKRNKIRE QNRYDLKTAG ----------
Clone 244S  TPIKIEDPNQ FVPLNTNPCV EG
```

319S

ACCAAAAGCTGATCGTGCAGATTTTAATAGAAAACGAAGATGTTCTGTTTTAGTCCATCAGGGAAGTGAGC
TGATAGAGCTAGTGGAATAAAAACGTTTCTCACACTTGGTTTGTCTTTTCAAACAAGTGGCAGAATTTCCT
GGACCACAGTGGATTGCAGAGTTGGGGACTGTGTCTCCCGCCTCCACATGAGAGCAAGGGTGAGGGGAGGA
AACCCCTTACCTTGGGTCTTTTGCCGTGCCTCGTATGTATGTCTGTTTCGCTGGAAGAGTGATTAATAAAT
CTTTCTTTAACTTATTAAAAAAATGTAGACTTTTAAGCTTCAGTCTTAACAGTAATAAAAGGGAACTTAAT
TGATAAAGGT

Comparison of clone 319S with AK026415

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319S:         1 accaaaagctgatcgtgcagattttaatagaaaacgaagatgttctgttttagtccatca 60
                ||||||||||||| |||||||||||||||||||||||||| ||| | || || |||||||
AK026415:  1346 accaaaagctgattgtgcagattttaatagaaaacgaagacgttttattctaatccatca 1405

319S:        61 gggaagtgagctgatagagctagt 84
                ||||| ||||||||
AK026415:  1406 gggaaatgagctga 1419


319S:        85 ggaataaaaacgtttctca-cacttggtttgtcttttcaaacaagtggcagaatttcctg 143
                |||||||||| ||||| | |||||| ||||| ||| ||| ||||||  ||||||| |||
AK026415:  1455 ggaataaaaacatttcttaccacttgatttgt-tttccaagcaagtgctagaatttgctg 1513

319S:       144 gaccacagtggattgcagagttggggactgtgtctcccg 182
                |||  ||| |||| || |||| ||| ||||||||||
AK026415:  1514 gactgcagaggatcgctgagtggggtactgtgtctc 1549


319S:       183 cctccacatgagagcaagggtgaggg-gagg-aaaccccttaccttgggtcttttgccgt 240
                ||||||| ||||| ||||||||| || |||| || ||||| ||||||||||||||||| ||
AK026415:  1564 cctccacgtgagaacaagggtgaaggtgagggaagcccctcaccttgggtcttttgctgt 1623

319S:       241 gcctcgtatgtatgtctgtttcgctggaagagtgattaataaatctttctttaacttatt 300
                ||||| |||||||||||| || ||||||||||||||||||| ||||||
AK026415:  1624 gcctcctatgtatgtctggtttgctggaagagtgattaatacatcttt 1671

319S        301 aaaaaaa 307
AK026415:


319S:       308 tgtagacttttaagcttcagtcttaacagtaataaaagggaacttaattgataaaggt 365
                ||||||| ||||| |||||||||||   |||||||||||||||||||| ||| ||||
AK026415:  1689 tgtagacctttaaacttcagtcttattggtaataaaagggaacttaattcatacaggt 1746
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Fig. 8. (A) Nucleotide sequences of clones 93S and 191S. Clones 93S and 191S were identified to be up-regulated by carrageenan inflammation by differential membrane hybridization and confirmed by ribonuclease protection assay. (B) Comparison of clones 244S and 319S with known sequences from Genbank. For clone 244S, the underlined sequence matched the rat γ-adducin sequence 1004–1626. The translated product of clone 244S was also compared with rat γ-adducin. For clone 319S, the underlined sequence matched the rat testis β-chimaerin sequence 1004–1107. In addition, a more extensive 3′ homology with human cDNA AK026415 was also observed.

$GABA_A$ receptor-associated protein kinase with high specificity for phosphorylating the β3 subunit has been isolated recently from calf brain membrane.[22] In view of the importance of the phosphorylation on GABA receptor function, the rat dorsal spinal cord may provide a good source to study the regulation of the β3 subunit-specific $GABA_A$ receptor-associated protein kinase and in turn the modulation of $GABA_A$ receptor activity.

The initial differential screening of the dorsal horn-enriched transcripts yielded a diverse group of gene products, only some of which are discussed below. Table 1 is confined to the known genes found in the Genbank non-redundant database. Two of these (23S, 372S) were not previously associated with nervous system function. Many of the genes identified in Table 1 represent signaling molecules that regulate the cytoskeleton (five of 12). Three of the identified genes are involved in the Rho GTPase pathway controlling cytoskeletal reorganization. Rho GTPases consisting of Rho, Rac and Cdc42 regulate actin changes leading to stress fibers, membrane ruffling and filopodia, respectively.[24] One of these genes, the *dbl*-proto-oncogene, functions as a guanine nucleotide exchange factor for the Rho GTPases (for review, see Quilliam *et al.*[39]). *Dbl* acts to convert Rho, Rac and Cdc42 to their GTP-bound active form, thereby stimulating cytoskeletal remodeling and membrane ruffling. Another of the identified genes (clone 319S) is a likely splice variant of β-chimaerin, because nucleotides 1–103 (underlined, Fig. 8B) matched rat β-chimaerin and, furthermore, could be translated to yield the same C-terminal portion of β-chimaerin. The sequence beyond 103 is likely a new 3′ untranslated region. The database search also showed a homology between 319S and the 3′ end of human β-2-chimaerin (no. U28926).[58] However, the longest homology was with a new human cDNA (no. AK026415; Fig. 8B). Translation of AK026415 yielded a protein with C-terminal homology to rat β- and human β-2-chimaerins from amino acids 193 (isoleucine) to 468 (phenylalanine) of human β-2-chimaerin, but with N-terminal divergence starting from amino acid 193. These data suggest that clone 319S may be the rat form of AK026415 and that it is a new, distinct β-chimaerin. The β-chimaerins and a related gene, N-chimaerin, represent Rho GTPase-activating proteins (RhoGAPs).[15,25] Like other RhoGAPs, β-chimaerin functions to accelerate the conversion of GTP-bound Rac to its GDP-bound state, thereby inactivating Rac. Since both Rho guanine nucleotide exchange factor and RhoGAP activities would stimulate Rho GTPase cycling, it is tempting to speculate that these two genes may work in concert to enhance stimulus-driven cytoskeletal remodeling in dorsal horn neurons.

The third gene, clone 244S, is highly homologous, but not identical to γ-adducin. The sequence 1–214 (underlined sequence in Fig. 8B) matched rat γ-adducin, while the remainder of the 3′ end (215–269) shared homology with several other proteins, but not with γ-adducin. Thus, it is possible that the 3′ end of 244S is a cloning artifact or that 244S is an isoform of γ-adducin. The sequence of clone 244S was within the protein coding sequence and translation of the clone yielded a C-terminal truncated γ-adducin which ended with the sequence, FVLNTNPCVEG; the C-terminal glycine is equivalent to leucine (amino acid 502) of γ-adducin, which is a considerably longer protein of 671 amino acids (see Fig. 8B, comparison of γ-adducin with translated result of clone 244S). The adducins are heteromeric and are involved in changes in cell morphology and cytoskeletal reorganization in response to external signals.[11] γ-Adducin is a membrane protein that binds actin filaments and thereby promotes the association of spectrin with F-actin. Interestingly, recent studies indicate that adducin is a major target of ROCK, a Rho-activated kinase. Activation of Rho stimulates ROCK to phosphorylate α-adducin, which enhances its F-actin binding activity and regulates membrane ruffling in cultured cells.[14] Taken together, these data highlight a role for the Rho–Rac family of cell shape genes in dorsal horn sensory processes. What function might an intracellular signaling system that controls membrane shape and cell motility perform in the dorsal horn? It has been demonstrated that activation of nociceptive primary afferents causes a marked change in dendritic morphology of noci-responsive second-order neurons. The tubular dendritic shafts take on a beaded appearance within 5 min of a strong nociceptive stimulus.[28] While the functional impact of this change is unclear, we hypothesize that stimulatory and inhibitory regulatory mechanisms in the Rho/Rac/CDC42 cascade are engaged to alter neuronal shape, and potentially functional neuronal plasticity during persistent pain states.

The dorsally enriched group of cytoskeletal-related proteins also included the PDZ domain-containing protein, synapse-associated protein 97 (SAP-97; clone 396S) and an isoform of rat opioid-binding cell adhesion molecule (Obcam; 88S). PDZ proteins play a role in receptor scaffolding and the molecular organization of receptor-signaling complexes.[17,36] It is noteworthy that the dorsal enrichment of SAP-97 is in very good agreement with the strong immunocytochemical staining for SAP-97 in laminae I/II, as reported previously.[33] Obcam was originally isolated as an opioid-binding protein.[26] Obcam is a member of a larger family of cell adhesion molecules that are anchored to the outer cell surface through a glycosyl phosphatidyl inositol linkage for cell–cell recognition, although some members may function as secreted signals.[23] This family also includes the neurotrimins and neuroplastins.[43,45] Neuroplastin 65 has been suggested to participate in hippocampal long-term potentiation, which has been cited as an analogous mechanism for long-term changes in spinal cord excitability in persistent pain states.[57]

When our subtracted library was screened by membrane hybridization for changes in gene expression induced by chronic noxious stimuli (carrageenan treatment), nine clones were found to be altered in two experiments; however, only three clones were confirmed further by ribonuclease protection assays. The problem of false-positives probably arose from technical factors. In order to hybridize as many clones as possible, only moderately stringent membrane hybridization conditions

were used, thereby allowing binding of family members that may not be regulated in the same fashion. The four clones regulated by carrageenan-induced noxious stimuli consisted of one known gene, cystatin C, and two unknown genes (Fig. 8A). The two unknown genes are currently being characterized more fully structurally and functionally.

Cystatin C, up-regulated by carrageenan inflammation, is a protein of 120 amino acids with cysteine protease inhibitory activity. Cystatin C is widely expressed in the CNS, as well as peripheral organs. It is secreted into biological fluids including cerebrospinal fluid, blood, saliva and urine.[5,48] In the normal rat brain, cystatin C is strongly expressed in astrocytes and weakly in a small number of neurons, and the cystatin C-positive astrocytes are localized preferentially in gray matter.[55] In agreement with this cellular distribution, cystatin C cDNA was not enriched in the dorsal spinal cord, as shown by the membrane hybridization screening (Table 1). Cystatin C gene expression is up-regulated by various stimuli. Enhanced cystatin C expression was observed following axotomy,[31] treatment with transforming growth factor-β[44] or dexamethasone.[1] Cystatin C is a highly efficient endogenous inhibitor of cysteine proteases and can regulate the proteolytic activity of endogenous and exogenous cysteine proteases. Interestingly, peripheral inflammation by formalin or zymosan,[46] or nerve injury by sciatic nerve ligation,[6] was found to activate spinal cord microglia and astrocytes. Furthermore, astrocytes are markedly activated in an osteosarcoma model of cancer pain.[42] Our provisional hypothesis is that cystatin C gene expression is up-regulated in, and secreted from, spinal astrocytes. However, the functional role of spinal cystatin C during persistent pain states is not readily obvious and requires further study.

As shown in Fig. 4, approximately one-third of cDNAs dotted on the membrane did not exhibit a detectable signal in the membrane screening hybridization. The problem may arise at least partially from the fact that the suppression-subtractive hybridization is known to isolate low-abundance transcripts, which are not easily detected by the membrane hybridization analysis. However, as shown in Fig. 6, the low-abundance clones (52S and 313S) are readily detected by the more sensitive RT–PCR analysis. Further study of the many unknown transcripts in this group (Fig. 4) may provide additional new pathways for understanding molecular functions in the dorsal spinal cord.

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